Detection of Occult Lymph Node Metastases in Esophageal Cancer by Minimally Invasive Staging Combined with Molecular Diagnostic Techniques

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ABSTRACT

Background and Objectives: Lymph node metastases are the most important prognostic factor in patients with esophageal cancer. Histologic examination misses micrometastases in up to 20% of lymph nodes evaluated. In addition, non-invasive imaging modalities are not sensitive enough to detect small lymph nodes metastases. The objective of this study was to investigate the use of reverse transcriptase-polymerase chain reaction (RT-PCR) of messenger RNA (mRNA) for carcinoembryonic antigen (CEA) to increase the detection of micrometastases in lymph nodes from patients with esophageal cancer.

Methods: RT-PCR of CEA mRNA was performed in lymph nodes from patients with malignant and benign esophageal disease. Each specimen was examined histopathologically and by RT-PCR and the results were compared.

Results: Metastases were present in 29 of 60 (48%) lymph nodes sample by minimally invasive staging from 13 patients with esophageal cancer when examined histopathologically. RT-PCR identified nodal metastases in 46 of these 60 (77%) samples. RT-PCR detected CEA mRNA in all 29 histologically positive samples and in 17 histologically negative lymph nodes. All lymph nodes from patients with benign disease (n=15) were negative both histopathologically and by RT-PCR. The stage of two patients was reclassified based on the RT-PCR results, which identified lymph node spread undetected histopathologically. Both of these patients developed recurrent disease after resection of the primary tumor.

Conclusions: RT-PCR is more sensitive than histologic examination in the detection of lymph node metastases in esophageal cancer and can lead to diagnosis of a more advanced stage in some patients. The combination of minimally invasive surgical techniques in combination with new molecular diagnostic techniques may improve our ability to stage cancer patients.

Key Words: Laparoscopy, Esophageal cancer, Lymph node metastasis.

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INTRODUCTION

The incidence of adenocarcinoma of the esophagus is rising at an alarming rate.[1] The prognosis is poor, with a five year survival of 5-10%. Randomized studies indicate that multimodality regimens have not been uniformly successful in improving survival compared to surgery alone.[2] One problem in the design of these trials is that pre- and post- treatment staging was performed by conventional imaging which has been shown to be inaccurate in up to 40% of patients with esophageal cancer when compared to minimally invasive surgical staging.[3,4] Even when surgical staging is performed, lymph node assessment may be inaccurate due to sampling errors at the time of nodal dissection.[3,4] In addition, micrometastases may escape detection by routine histopathologic methods in up to 20% of cases.[5] The application of recent advances in molecular biology may facilitate the detection of micrometases in lymph nodes, thereby limiting the requirement for extensive surgical sampling, and increasing the accuracy of staging.

Carcinoembryonic antigen (CEA) is a recognized tumor marker that is expressed in a variety of malignancies. CEA has been most extensively studied as a serum marker of recurrence of colorectal cancer after surgical resection.[6] The complete gene for CEA has recently been cloned and complete sequence information is available.[7] Recently, a CEA-specific nested PCR assay to detect CEA producing cells from bone marrow aspirates has been described.[8] In a preliminary study, this assay was modified to detect micrometastases in lymph nodes in a small number of patients with esophageal, gastric, colorectal, and breast carcinoma.[9]

The purpose of this investigation was to investigate the utility of RT-PCR for detecting micrometastases in histologically negative lymph nodes in patients with esophageal cancer. The ability to detect micrometastases by utilizing minimally invasive surgical techniques would provide concrete pathological evidence of lymph node status, without subjecting patients to unnecessary surgical intervention. Minimally invasive surgical techniques have been shown to be cost-effective as well as to improve patient outcomes, and in the application of lymph node sampling for staging purposes, this modality would ultimately serve as a "gold standard."

MATERIALS AND METHODS

RNA Extraction

Total cellular RNA was extracted from all samples by a modified guanidinium thiocyanate extraction method[10] using a RNA isolation kit (RNeasy, Qiagen, Chatsworth, CA). Tissue lysis and homogenization were performed with an automated tissue homogenizer (PowerGen 35 Homogenizer, Fisher Scientific), using disposable generator tips to prevent RNA cross contamination.

Reverse Transcription

cDNA was synthesized from 1-5 µg of total RNA using a cDNA synthesis kit (Superscript preamplification system, GibcoBRL, Gaithersburg, MD). The oligo-dT priming method was utilized.

Polymerase Chain Reaction

Three CEA specific oligonucleotide primers for nested PCR were designed from previously published sequences.[7-9] The primer sequences for CEA were as follows: Primer A, 5'-TCTGGAACTTCTCCTG-GTCTCTCAGCTGG-3'; Primer B, 5'-TGTAGCTGTTG-CAAATGCTTTAAGGAAGAAGC-3'; and Primer C, 5'-GGGCCACTGTCGGCATCATGATTGG-3'. The nested PCR was performed in two successive steps. Step 1 was performed in a 20 µl reaction volume containing 1X PCR buffer (Boehringer Mannheim), 250 µM dNTPs, 1 µM each primers A and B, and 0.5 U Taq DNA polymerase (Boehringer Mannheim). Twenty rounds of amplification were performed in a thermocycler (DNA Thermocycler 480, Perkin Elmer) under the following cycling conditions: 95° C (1 min) denaturation, 72° C (1

Table 1.
Results of histological and RT-PCR examination of lymph nodes from patients with esophageal carcinoma.

| Case number | Diagnosis | Positive Lymph Nodes | | Status/Follow-up |
		Histology	RT-PCR	
1	Adeno	0/2	2/2	DWD/5 mos
2	Adeno	0/1	1/1	DWD/2 mos
3	Adeno	0/2	0/2	NED/12 mos
4	Adeno	2/4	3/4	NED/11 mos
5	Adeno	6/8	7/8	AWD/7 mos
6	Adeno	7/17	12/17	AWD/9 mos
7	Adeno	5/8	8/8	NED/7 mos
8	SCC	1/7	3/7	AWD/7 mos
9	Adeno	1/2	2/2	AWD/20 mos
10	Adeno	1/2	2/2	AWD/7 mos
11	Adeno	3/4	3/4	DWD/10 mos
12	Adeno	1/1	1/1	AWD/9 mos
13	Adeno	2/2	2/2	NED/5 mos

Abbreviations: Adeno=esophageal adenocarcinoma; SCC=esophageal squamous cell carcinoma; DWD=died with disease; NED=no evidence of disease; AWD=alive with disease

min) annealing, 72° C (1 min) extension, followed by a final extension of 72° C (10 min). Two µl of the Step 1 reaction product was transferred to another tube containing primers B and C, PCR buffer, dNTPs, and Taq DNA polymerase in the same concentrations as in Step 1. Step 2 was also performed in a 20 µl reaction volume. The second PCR reaction was performed under the same conditions as Step 1. Step 1 yields a 160-bp PCR product; the second PCR reaction produces a 131-bp product.

As a positive control for RNA quality, a PCR assay for â-actin was performed for each sample. The primer sequences were as follows: 5'-CCTGGCACCCAGCA-CAATGA-3', and 5'-ACGAAGGCTCATCATTCAAA-3'. The actin PCR was performed with 30 rounds of amplification under the following cycling conditions: 94° C (30 sec) denaturation, 68° C (45 sec) annealing, 72° C (1 min) extension, followed by a 7 minute final extension at 72° C.

To check for possible contamination with genomic DNA, RT-PCR reactions were also performed without a reverse transcription step.

PCR Product Analysis

PCR products were analyzed on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide. A 100-bp ladder (Boehringer Mannheim) served as a molecular weight marker. PCR products were separated by electrophoresis at 80 volts for 2-3 h.

Lymph Node Samples

Informed consent was obtained from all patients as specified in University of Pittsburgh Cancer Institute protocol 94-121. Sixty lymph node samples were obtained from 13 patients with carcinoma of the esophagus. Lymph nodes from patients with esophageal cancer were obtained during combined laparoscopic and videothoracoscopic staging or esophagectomy. As a control, 10 lymph nodes were obtained from 6 patients undergoing treatment of a benign esophageal disease, such as gastroesophageal reflux disease or achalasia. Five lymph nodes were evaluated from a patient with a history of long-standing reflux and Barrett's metaplasia. In addition to lymph nodes, 8 samples of esophageal carcinoma (7 adenocarcinoma, 1 squamous cell carcinoma), and 5 samples of normal esophagus were studied.

Each sample was carefully dissected from perinodal fat

and soft tissue and then sectioned into two pieces. One piece was fixed and embedded in paraffin and used for histologic analysis. The remaining piece was immediately placed in liquid nitrogen and stored at -70°C until RNA extraction. Following the RT-PCR analysis the results of histologic examination and RT-PCR were compared.

RESULTS

Normal Esophagus and Carcinoma Samples

RT-PCR amplification of all 8 samples of esophageal carcinoma and 5 samples of normal esophagus demonstrated the 131-bp CEA band **(Figure 1)**. No product was observed in control PCR reactions performed without the reverse transcription, demonstrating that there was no contamination with genomic DNA **(Figure 2)**.

Lymph Node Samples

Histologic examination detected metastases in 29 of 60 (48%) lymph node specimens studied. All histologically positive lymph node samples demonstrated a positive CEA signal by RT-PCR. The number of lymph node metastases detected increased to 46 of 60 (77%) when RT-PCR was used. Seventeen of 31 (52%) histologically
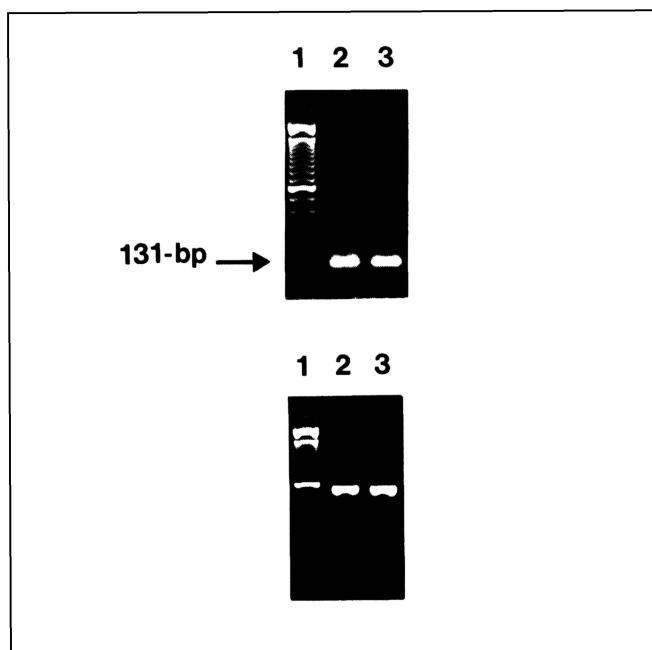

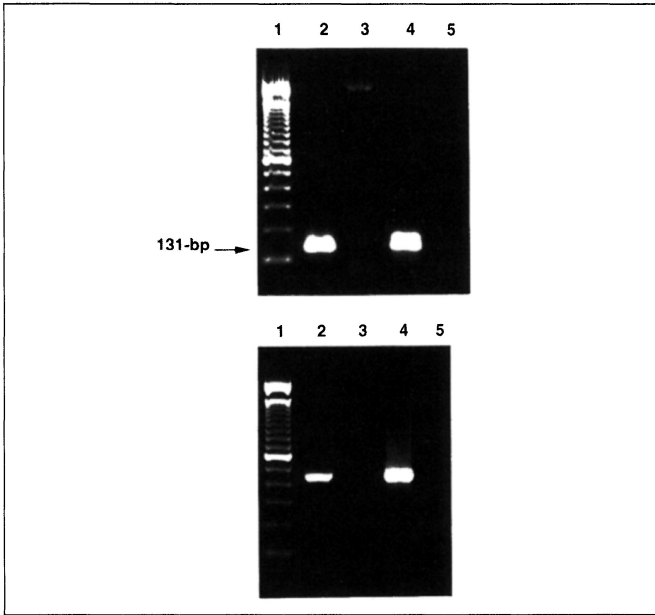**Figure 1.** Occult lymph node metastases in esophageal cancer.

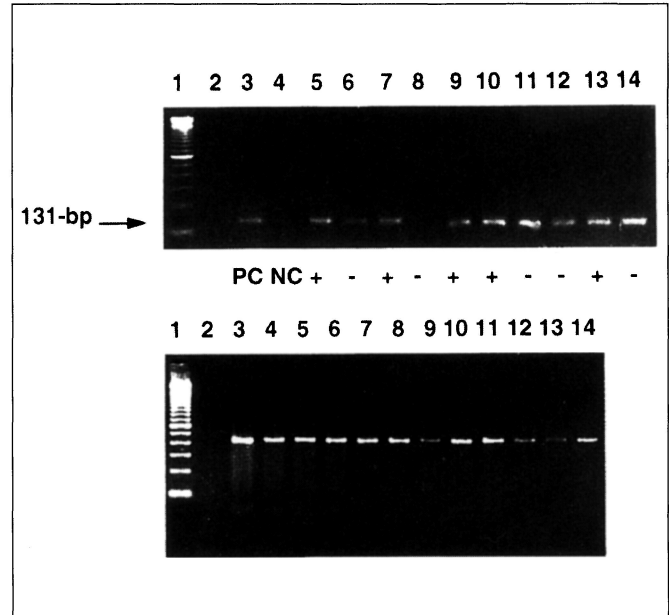**Figure 2.** Occult lymph node metastases in esophageal cancer.

Figure 3. Occult lymph node metastases in esophageal cancer.

negative lymph nodes were found by RT-PCR analysis to express the mRNA for CEA by RT-PCR **(Figure 3)**. In three patients, all regional lymph nodes were negative histologically. Of these patients, two demonstrated lymph node metastases by RT-PCR and both of these patients died of disease progression within 12 months. The remaining patient, negative histologically and by RT-PCR, remains without disease recurrence 12 months after surgical resection. In nine patients, RT-PCR indicated an increased number of metastatic lymph nodes compared to histologic review. In three patients, histologic and RT-PCR diagnostic methods detected the same number of lymph node metastases **(Table 1)**.

All lymph nodes with histologic evidence of metastases failed to demonstrate the characteristic 131-bp band when PCR without reverse transcription was performed, indicating that genomic DNA contamination did not occur. All lymph nodes from patients with benign esophageal diseases and Barrett's esophagus were negative for CEA by RT-PCR and negative by histologic examination.

DISCUSSION AND CONCLUSIONS

The identification of lymph node metastasis in patients with esophageal cancer is an important determinant of prognosis and impacts on the clinical decision making process.[11-13] Randomized trials of multimodality treatment regimens have been performed but have not uniformly improved survival compared to surgery alone.[2] A major drawback in these studies is that accurate pretreatment staging prior to the initiation of therapy was not performed, thus limiting any meaningful evaluation of therapeutic response. This approach of grouping patients based only on the presence or absence of distant metastases without consideration of the extent of locoregional disease also limits evaluation of survival data on a stage by stage basis. Since a number of reports have shown that not only the presence of lymph node metastases but their number and location are important indicators of long-term survival, it is possible that a more accurate assessment of stage prior to treatment would allow a more thorough evaluation of efficacy.[11-13]

Conventional imaging modalities employed to assess lymph node and distant metastases are inaccurate in over 40% of patients when compared to minimally invasive surgical staging.[3,4] Positron emission tomography offers

a significant improvement over computerized tomography in detecting distant metastatic disease but is only 45% sensitive in detecting metastases lymph nodes under 1 cm in diameter.[4] Endoscopic ultrasound is accurate in assessment of the depth of tumor invasion but its utility in detecting small lymph node metastases has been questioned.[14-17]

Currently, the most accurate method to stage patients with esophageal cancer is by minimally invasive surgical biopsy. However, this approach is associated with a small but measurable morbidity, significant expense and requires an extensive surgical dissection to avoid sampling errors and under staging. Once lymph nodes are surgically biopsied, further errors may take place in the histologic evaluation since up to 20% of histologically negative lymph nodes are positive on re-examination.[5] Immunohistochemical staining of lymph nodes to detect tumor markers may result in a higher detection rate of micrometastases when compared to histological examination but this technique is time consuming and labor intensive, and is not in widespread clinical use.[18-21]

Sensitive molecular methods, such as RT-PCR, have recently been employed to detect lymph node metastases in a variety of malignancies such as malignant melanoma, breast, colorectal, gastric, and esophageal carcinoma.[9,22-25] This method is designed to detect markers expressed in the carcinoma cells but not in normal lymph nodes. A CEA specific RT-PCR assay has been developed to detect carcinoma cells in bone marrow samples from patients with colorectal, pancreatic, or gastric carcinoma.[8] Recently, this method has been modified to detect lymph nodes metastases in patients with esophageal, gastric, colorectal, and breast cancer.[9] This preliminary report included a small number of patients with esophageal cancer.

Our preliminary experience with RT-PCR to detect the mRNA for CEA confirms that this technique has the potential to improve the accuracy of detecting lymph node metastases in esophageal cancer compared to histologic evaluation. As expected, CEA was present in all samples of esophageal carcinoma and normal esophagus and thus appears to be a marker specific for epithelial cells. Importantly, CEA was not present in any of the lymph nodes from patients without cancer. Thus in the absence of cancer there is no migration of epithelial cells from the esophagus to the lymph nodes. Histologic assessment of lymph nodes from patients with esophageal cancer detected 29 of 60 lymph nodes

involved with metastatic disease. All histologically positive lymph nodes were positive by RT-PCR for CEA, indicating that there were no false negative results using this method. The number of positive lymph nodes increased to 49 of 60 when RT-PCR technology was applied. Two of three patients with histologically negative lymph nodes were determined to have nodal disease by RT-PCR staging. In nine of the 13 patients with esophageal cancer, the number of positive lymph nodes detected was increased by RT-PCR compared to histology.

CEA-specific RT-PCR is an extremely sensitive technique that facilitates the detection of histologically occult lymph node metastases in patients with esophageal cancer. The technique has the potential to improve upon the accuracy of current staging, aid in the evaluation of response to treatment, and impact on clinical decision making in patients with esophageal cancer. Further studies with clinical follow-up will be required to evaluate the role of RT-PCR in the diagnosis and treatment of patients with esophageal cancer.

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